(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2002 (03.01.2002)

PCT

(10) International Publication Number
WO 02/00927 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/EP01/07536

(22) International Filing Date: 2 July 2001 (02.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 32 529.7 30 June 2000 (30.06.2000) DE
100 43 826.1 1 September 2000 (01.09.2000) DE

(71) Applicant *(for all designated States except US)*: **EPIGENOMICS AG** [DE/DE]; Kastanienallee 24, 10435 Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **OLEK, Alexander** [DE/DE]; Schröderstrasse 13/2, 10115 Berlin (DE). **PIEPENBROCK, Christian** [DE/DE]; Schwartzkopffstrasse 7 B, 10115 Berlin (DE). **BERLIN, Kurt** [DE/DE]; Marienkäferweg 4, 14532 Stahnsdorf (DE).

(74) Agents: **SCHOHE, Stefan** et al.; Boehmert & Boehmert, Franz-Joseph-Strasse 38, 80801 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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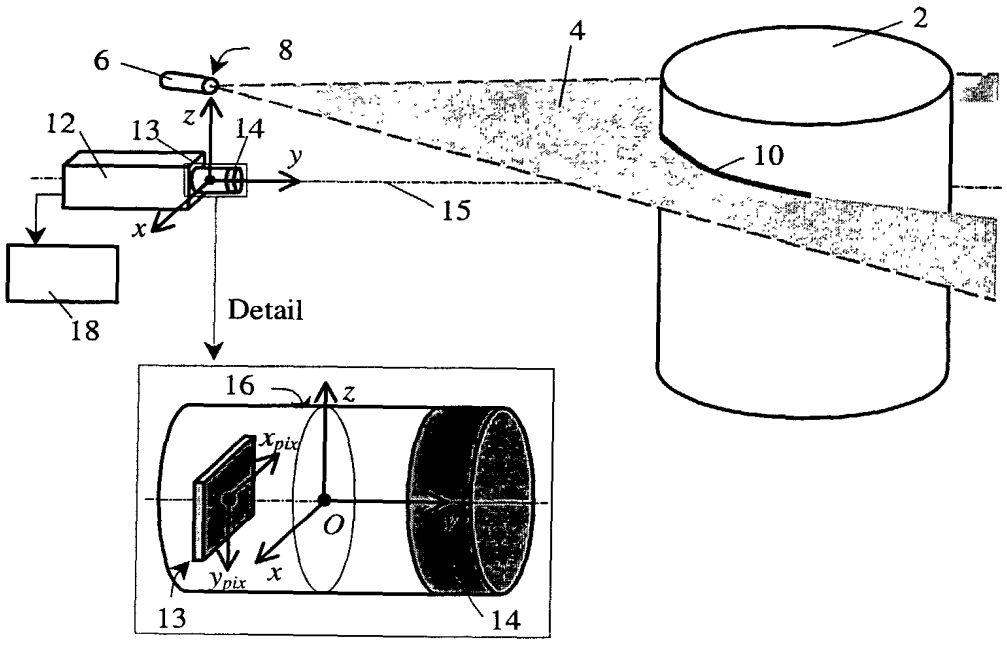
(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH DEVELOPMENT GENES



(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with diseases associated with development, to oligonucleotides and/or PNA/oligomers for detecting the cytosine methylation state of genes associated with diseases associated with development which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with diseases associated with development.

WO 02/00927 A2

Published:

— *without international search report and to be republished upon receipt of that report*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Diagnosis of diseases associated with development genes

Field of the Invention

The levels of observation that have been well studied by the methodological developments of recent years in molecular biology, are the genes themselves, the translation of these genes into RNA, and the resulting proteins. The question of which gene is switched on at which point in the course of the development of an individual, and how the activation and inhibition of specific genes in specific cells and tissues are controlled is correlatable to the degree and character of the methylation of the genes or of the genome. In this respect, pathogenic conditions may manifest themselves in a changed methylation pattern of individual genes or of the genome.

The present invention relates to nucleic acids, oligonucleotides, PNA-oligomers and to a method for the diagnosis and/or therapy of diseases which have a connection with the genetic and/or epigenetic parameters of genes associated with development and, in particular, with the methylation status thereof.

Prior Art

The ordered progression of embryonic development is controlled by a complex genetic regulatory system. Initial identification of key genes involved in development were in model organisms such as Drosophila. This was followed by the identification of highly conserved human homologues e.g. the frizzled gene and its human homologue. Examples of important transcription factors involved in development include the homeobox, MAD, PAX, EMX and MSX gene families (see, e.g. Terzic J, Saraga-Babic M.. Expression pattern of PAX3 and PAX6 genes during human embryogenesis. Int J Dev Biol 1999 Sep;43(6):501-8)

Genes involved in development have been implicated in a wide range of diseases. For example, the homeobox containing genes (HOX), are primarily involved in normal development. However, the HOX genes have also been implicated in normal adult cellular functioning and a wide range of disease including diabetes and cancer ('Homeobox genes in normal and ma-

lignant cells' Cillo C *et. al.* J Cell Physiol 2001 Aug;188(2):161-9. 'Homeobox genes in leukemogenesis' Buske C and Humphries RK Int J Hematol 2000 Jun;71(4):301-8. 'HOX genes in human cancers.' Cillo C Invasion Metastasis 1994-95;14(1-6):38-49). Other developmental related diseases comprise apoptosis related diseases (see, e.g.Sano M, et al. Involvement of EAT/mcl-1, an anti-apoptotic bcl-2-related gene, in murine embryogenesis and human development. Exp Cell Res 2000 Aug 25;259(1):127-39; Muller S, et al. Retention of imprinting of the human apoptosis-related gene TSSC3 in human brain tumors. Hum Mol Genet 2000 Mar 22;9(5):757-63), syndromes associated with congenital heart disease (see, e.g. Gelb BD. Genetic basis of syndromes associated with congenital heart disease. Curr Opin Cardiol 2001 May;16(3):188-94) epilepsy (see, e.g. Doose H, Neubauer BA, Petersen B. The concept of hereditary impairment of brain maturation. Epileptic Disord 2000;2 Suppl 1:S45-9, diseases related to histone deacetylation (see, e.g. El-Osta A, Wolffe AP. DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease. Gene Expr 2000;9(1-2):63-75) Currarino syndrome (see e.g. Hagan DM, et al. Mutation analysis and embryonic expression of the HLXB9 Currarino syndrome gene. Am J Hum Genet 2000 May;66(5):1504-15 Erratum in: Am J Hum Genet 2000 Sep;67(3):769), diseases related with the development of the brain and limb girdle muscular dystrophy (Wnt7a and CAPN3, see, e.g. Fougerousse F, et al. Human-mouse differences in the embryonic expression patterns of developmental control genes and disease genes. Hum Mol Genet 2000 Jan 22;9(2):165-73; Hum Mol Genet 2000 Mar 1;9(4):659), dwarfism (see, e.g. Iwata T, et al. Highly activated Fgfr3 with the K644M mutation causes prolonged survival in severe dwarf mice. Hum Mol Genet 2001 Jun 1;10(12):1255-1264), and others (see, e.g. Hanel ML, Wevrick R. The role of genomic imprinting in human developmental disorders: lessons from Prader-Willi syndrome. Clin Genet 2001 Mar;59(3):156-64).

5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis.

The importance of methylation in developmental genes has been well established. Aberrant genomic imprinting (parental origin specific methylation) results in abnormal embryonic development. For example, in mice the IGF2 gene is differentially methylated according to the parental origin of the allele. The allele is thought ot be relevant during late embryonic devel-

opment and act to regulate growth. Aberrant methylation results in gross abnormalities, the resultant foetus being unviable, or dying at birth. Furthermore, this gene has been shown to be significant in the development of human cancers and growth disorders such as Beckwith–Wiedemann (Transactivation of Igf2 in a mouse model of Beckwith–Wiedemann syndrome. Sun *et. al.* Nature 389, 809 - 815. 1997).

Aberrant DNA methylation within CpG islands is common in human malignancies leading to abrogation or overexpression of a broad spectrum of genes (Jones, P.A. Cancer Res 65:2463-2467, 1996). Abnormal methylation has also been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours (Chan, M.F., et al., Curr Top Microbiol Immunol 249:75-86,2000). Using restriction landmark genomic scanning, Costello and coworkers were able to show that methylation patterns are tumour-type specific (Costello, J. F., et al., Nat Genet 24:132-138, 2000). Highly characteristic DNA methylation patterns could also be shown for breast cancer cell lines (Huang, T. H.-M., et al., Hum Mol Genet 8:459-470, 1999). Genome wide assessment of methylation status represents a molecular fingerprint of cancer tissues.

Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing development as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analyzing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behaviour. However, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridization behaviour, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridization or sequencing. All of these techniques are based on base pairing which can now be fully exploited. In terms of sensitivity, the prior art is defined by a method which encloses the DNA to be analyzed in an agarose matrix, thus pre-

venting the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and which replaces all precipitation and purification steps with fast dialysis (Olek A, Oswald J, Walter J. A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res. 1996 Dec 15;24(24):5064-6). Using this method, it is possible to analyze individual cells, which illustrates the potential of the method. However, currently only individual regions of a length of up to approximately 3000 base pairs are analyzed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyze very small fragments from small sample quantities either. These are lost through the matrix in spite of the diffusion protection.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., Nucleic Acids Res. 1998, 26, 2255.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet. 1997 Mar-Apr;5(2):94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. Nat Genet. 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalgo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res. 1997 Jun 15;25(12):2529-31, WO 95/00669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 1997 Jun 15;25(12):2532-4). In addition, detection by hybridization has also been described (Olek et al., WO 99/28498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. Bioessays. 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic

sequencing method. Hum Mol Genet. 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res. 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. Gene. 1995 May 19;157(1-2):261-4; WO 97/46705, WO 95/15373 and WO 97/45560.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (Nature Genetics Supplement, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorescently labeled probes are often used for the scanning of immobilized DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridized probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapor phase in an unfragmented manner. The analyte is ionized by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Current Innovations and Future Trends. 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse than to peptides and decreases disproportionally with increasing fragment size. For nucleic

acids having a multiply negatively charged backbone, the ionization process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallization. There are now several responsive matrixes for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. Nucleic Acids Res. 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989.

Description

The object of the present invention is to provide the chemically modified DNA of genes associated with diseases associated with development genes, as well as oligonucleotides and/or PNA-oligomers for detecting cytosine methylations, as well as a method which is particularly suitable for the diagnosis and/or therapy of genetic and epigenetic parameters of genes associated development, and diseases associated with those genes. The present invention is based on the discovery that genetic and epigenetic parameters and, in particular, the cytosine methylation pattern of genes associated with development are particularly suitable for the diagnosis and/or therapy of diseases.

This objective is achieved according to the present invention using a nucleic acid containing a sequence of at least 18 bases in length of the chemically pretreated DNA of genes associated with development according to one of Seq. ID No.1 through Seq. ID No.350 and sequences

complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1. In the table, after the listed gene designations, the respective data bank numbers (accession numbers) are specified which define the appertaining gene sequences as unique. GenBank was used as the underlying data bank which is located at internet address http://www.ncbi.nlm.nih.gov

The chemically modified nucleic acid could heretofore not be connected with the ascertainment of genetic and epigenetic parameters.

The object of the present invention is further achieved by an oligonucleotide or oligomer for detecting the cytosine methylation state in chemically pretreated DNA, containing at least one base sequence having a length of at least 13 nucleotides which hybridizes to a chemically pretreated DNA of genes associated with development according to Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1. The oligomer probes according to the present invention constitute important and effective tools which, for the first time, make it possible to ascertain the genetic and epigenetic parameters of genes associated with development. The base sequence of the oligomers preferably contains at least one CpG dinucleotide. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Particularly preferred are oligonucleotides according to the present invention in which the cytosine of the CpG dinucleotide is the 5^{th} - 9^{th} nucleotide from the 5'-end of the 13-mer; in the case of PNA-oligomers, it is preferred for the cytosine of the CpG dinucleotide to be the 4^{th} - 6^{th} nucleotide from the 5'-end of the 9-mer.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for each of the CpG dinucleotides of the sequences of Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides from one of Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of

genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1.

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one oligonucleotide is bound to a solid phase. It is further preferred that all the oligonucleotides of one set are bound to a solid phase.

The present invention moreover relates to a set of at least 10 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in chemically pretreated genomic DNA (Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with development, and associated diseases. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in the chemically pretreated DNA of genes associated with diseases associated with development according to one of Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1.

According to the present invention, it is preferred that an arrangement of different oligonucleotides and/or PNA-oligomers (a so-called "array") made available by the present invention is present in a manner that it is likewise bound to a solid phase. This array of different oligonucleotide- and/or PNA-oligomer sequences can be characterized in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of

pellets or also as resin matrices are possible as well.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a carrier material for analysis in connection with diseases associated with development genes in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

A further subject matter of the present invention relates to a DNA chip for the analysis of diseases associated with development genes which contains at least one nucleic acid according to the present invention. DNA chips are known, for example, for US Patent 5,837,832.

Moreover, a subject matter of the present invention is a kit which may be composed, for example, of a bisulfite-containing reagent, a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond or are complementary to an 18 base long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1.), oligonucleotides and/or PNA-oligomers as well as instructions for carrying out and evaluating the described method. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

The present invention also makes available a method for ascertaining genetic and/or epigenetic parameters of development genes associated with diseases by analyzing cytosine methylations and single nucleotide polymorphisms, including the following steps:

In the first step of the method, a genomic DNA sample is chemically treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or another base which is dissimilar to cytosine in terms of hybridization behaviour. This will be understood as 'chemical pretreatment' hereinafter.

The genomic DNA to be analyzed is preferably obtained form usual sources of DNA such as

cells or cell components, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, or combinations thereof.

The above described treatment of genomic DNA is preferably carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behaviour.

Fragments of the chemically pretreated DNA are amplified, using sets of primer oligonucleotides according to the present invention, and a, preferably heat-stable polymerase. Because of statistical and practical considerations, preferably more than ten different fragments having a length of 100 - 2000 base pairs are amplified. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel. Usually, the amplification is carried out by means of a polymerase chain reaction (PCR).

In a preferred embodiment of the method, the set of primer oligonucleotides includes at least two oligonucleotides whose sequences are each reverse complementary or identical to an at least 18 base-pair long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1). The primer oligonucleotides are preferably characterized in that they do not contain any CpG dinucleotides.

According to the present invention, it is preferred that at least one primer oligonucleotide is bonded to a solid phase during amplification. The different oligonucleotide and/or PNA-oligomer sequences can be arranged on a plane solid phase in the form of a rectangular or hexagonal lattice, the solid phase surface preferably being composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold, it being possible for other materials such as nitrocellulose or plastics to be used as well.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detach-

able molecule fragments having a typical mass which can be detected in a mass spectrometer, it being preferred that the fragments that are produced have a single positive or negative net charge for better detectability in the mass spectrometer. The detection may be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The amplificates obtained in the second step of the method are subsequently hybridized to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridization takes place in the manner described in the following. The set of probes used during the hybridization is preferably composed of at least 10 oligonucleotides or PNA-oligomers. In the process, the amplificates serve as probes which hybridize to oligonucleotides previously bonded to a solid phase. The non-hybridized fragments are subsequently removed. Said oligonucleotides contain at least one base sequence having a length of 13 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 5^{th} to 9^{th} nucleotide from the 5'-end of the 13-mer. One oligonucleotide exists for each CpG dinucleotide. Said PNA-oligomers contain at least one base sequence having a length of 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 4^{th} to 6^{th} nucleotide seen from the 5'-end of the 9-mer. One oligonucleotide exists for each CpG dinucleotide.

In the fourth step of the method, the non-hybridized amplificates are removed.

In the final step of the method, the hybridized amplificates are detected. In this context, it is preferred that labels attached to the amplificates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

According to the present invention, it is preferred that the labels of the amplificates are fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer. The mass spectrometer is preferred for the detection of the amplificates, fragments of the amplificates or of probes which are complementary to the amplificates, it being possible for the detection to be carried out and visualized by means

of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The produced fragments may have a single positive or negative net charge for better detectability in the mass spectrometer. The aforementioned method is preferably used for ascertaining genetic and/or epigenetic parameters of genes associated with development and diseases.

The oligomers according to the present invention or arrays thereof as well as a kit according to the present invention are intended to be used for the diagnosis and/or therapy of diseases associated with development genes by analyzing methylation patterns of developmental genes associated with diseases. According to the present invention, the method is preferably used for the diagnosis and/or therapy of important genetic and/or epigenetic parameters within developmental genes associated with diseases.

The method according to the present invention is used, for example, for the diagnosis and/or therapy of diseases associated with development genes.

The nucleic acids according to the present invention of Seq. ID No.1 through Seq. ID No.350 and sequences complementary thereto and/or a chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes according to table 1 can be used for the diagnosis and/or therapy of genetic and/or epigenetic parameters of development genes associated with diseases.

The present invention moreover relates to a method for manufacturing a diagnostic agent and/or therapeutic agent for the diagnosis and/or therapy of diseases associated with development by analyzing methylation patterns of genes associated with diseases associated with development, the diagnostic agent and/or therapeutic agent being characterized in that at least one nucleic acid according to the present invention is used for manufacturing it, possibly together with suitable additives and auxiliary agents.

A further subject matter of the present invention relates to a diagnostic agent and/or therapeutic agent for diseases associated with development genes by analyzing methylation patterns of development genes associated with diseases, the diagnostic agent and/or therapeutic agent

containing at least one nucleic acid according to the present invention, possibly together with suitable additives and auxiliary agents. Such diseases associated with development are, e.g. diseases related to homeobox containing genes (HOX), e.g. diabetes and cancer, apoptosis related diseases, syndromes associated with congenital heart disease, epilepsy, diseases related to histone deacetylation, Currarino syndrome, diseases related with the development of the brain and limb girdle muscular dystrophy, dwarfism, and others.

The present invention moreover relates to the diagnosis and/or prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within development genes associated with diseases, said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a diagnosis and/or prognosis of events which are disadvantageous to patients or individuals. Such diseases associated with development are, e.g. diseases related to homeobox containing genes (HOX), e.g. diabetes and cancer, apoptosis related diseases, syndromes associated with congenital heart disease, epilepsy, diseases related to histone deacetylation, Currarino syndrome, diseases related with the development of the brain and limb girdle muscular dystrophy, dwarfism, and others.

In the context of the present invention the term "hybridization" is to be understood as a bond of an oligonucleotide to a completely complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure. To be understood by "stringent hybridization conditions" are those conditions in which a hybridization is carried out at 60°C in 2.5 x SSC buffer, followed by several washing steps at 37°C in a low buffer concentration, and remains stable.

The term "functional variants" denotes all DNA sequences which are complementary to a DNA sequence, and which hybridize to the reference sequence under stringent conditions and have an activity similar to the corresponding polypeptide according to the present invention.

In the context of the present invention, "genetic parameters" are mutations and polymorphisms of development genes associated with diseases and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide

polymorphisms).

In the context of the present invention, "epigenetic parameters" are, in particular, cytosine methylations and further chemical modifications of DNA bases of genes associated with diseases associated with development and sequences further required for their regulation. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analyzed using the described method but which, in turn, correlates with the DNA methylation.

In the following, the present invention will be explained in greater detail on the basis of the sequences and examples with reference to the accompanying drawing, without being limited thereto.

Figure 1

Figure 1 shows the hybridisation of fluorescent labelled amplificates to a surface bound olignonucleotide. Flourescence at a spot shows hybridisation of the amplificate to the oligonucleotide. Hybridisation to a CG olignonucleotide denotes methylation at the cytosine position being analysed, hybridisation to a TG olignonucleotide denotes no methylation at the cytosine position being analysed. It can be seen that Sample II had a higher degree of methylation than Sample I.

Seq. ID No. 1 trough Seq. ID No. 350

Sequences having odd sequence numbers (e.g., Seq. ID No. 1, 3, 5, ...) exhibit in each case sequences of the chemically pretreated genomic DNAs of different genes associated with diseases associated with development. Sequences having even sequence numbers (e.g., Seq. ID No. 2, 4, 6, ...) exhibit in each case the sequences of chemically pretreated genomic DNAs. Said genomic DNAs are complementary to the genomic DNAs from which the preceding sequence was derived (e.g., the complementary sequence to the genomic DNA from which Seq. ID No.1 is derived is the genomic sequence from which Seq. ID No.2 is derived, the complementary sequence to the genomic DNA from which Seq. ID No.3 is derived is the sequence from which Seq. ID No.4 is derived, etc.)

Seq. ID No. 351 trough Seq. ID No. 354

Seq. ID No. 351 trough Seq. ID No. 354 show sequences of oligonucleotides used in Example 1.

Example 1 :Methylation analysis of the gene PBX2.

The following example relates to a fragment of the gene PBX2 in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturating reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene PBX2 are analyzed. To this end, a defined fragment having a length of 718 bp is amplified with the specific primer oligonucleotides GTTTTTAGAAGATTTAGAATATGTG (Sequence ID 47) and CCACTAAATCTCAATTCCTCT (Sequence ID No. 48). The single gene PCR reaction was performed on a thermocycler (Eppendorf GmbH) using bisulfite DNA 10 ng, primer 6 pmole each, dNTP 200 µM each, 1.5 mM MgCl2 and 1 U HotstartTaq (Qiagen AG). The other conditions were as recommended by the Taq polymerase manufacturer. In the multiplex PCR up to 16 primer pairs were used within the PCR reaction. The multiplex PCR was done according the single gene PCR with the following modifications: primer 0.35 pmole each, dNTP 800 µM each and 4,5 mM MgCl2. The cycle program for single gene PCR and multiplex PCR was as followed: step 1,14 min 96 °C; step 2, 60 sec 96°C; step 3, 45 sec 55 °C; step 4 ,75 sec 72 °C; step 5, 10 min 72 °C; the step 2 to step 4 were repeated 39 fold.

The amplificate serves as a sample which hybridizes to an oligonucleotide previously bound

to a solid phase, forming a duplex structure, for example TGGGATATCGGTTGGGTT (Sequence ID No. 49), the cytosine to be detected being located at position 470 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said olignonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e TGGGATATTGGTTGGGTT (Sequence ID No. 50). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 2: Diagnosis of diseases associated with development genes

In order to relate the methylation patterns to one of the diseases associated with development, it is initially required to analyze the DNA methylation patterns of a group of diseased and of a group of healthy patients. These analyses are carried out, for example, analogously to Example 1. The results obtained in this manner are stored in a database and the CpG dinucleotides which are methylated differently between the two groups are identified. This can be carried out by determining individual CpG methylation rates as can be done, for example, in a relatively imprecise manner, by sequencing or else, in a very precise manner, by a methylation-sensitive "primer extension reaction". It is particularly preferred that the determination be carried out in the manner described in Example 1, bisulphite treatment of genomic DNA followed by fluorescence hybridisation analysis on an oligomer array, thereby enabling the simultaneous analysis of multiple positions within the genome. It is also possible for the entire methylation status to be analyzed simultaneously, and for the patterns to be compared, for example, by clustering analyses which can be carried out, for example, by a computer.

Subsequently, it is possible to allocate the examined patients to a specific therapy group and to treat these patients selectively with an individualized therapy.

Example 2 can be carried out, for example, for diseases associated with development, such as diseases related to homeobox containing genes (HOX), e.g. diabetes and cancer, apoptosis related diseases, syndromes associated with congenital heart disease, epilepsy, diseases related to histone deacetylation, Currarino syndrome, diseases related with the development of the brain and limb girdle muscular dystrophy, dwarfism, and others.

Table 1

List of preferred genes associated with development according to the invention

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
ACCPN	n.a.*
ADFN	n.a.*
AFD1	n.a.*
AHO2	n.a.*
AIH3	n.a.*
AMCD1	n.a.*
AMCD2B	n.a.*
AMCN	n.a.*
AMCX1	n.a.*
AMDM	n.a.*
ANOP1	n.a.*
ASMD	n.a.*
ATD	n.a.*
AXIN1	n.a.*
AF009674	n.a.*
BDB1	n.a.*
BDC	n.a.*
BDE	n.a.*
BDMR	n.a.*
CHH	n.a.*
CHRD	AF076612
CHX10	n.a.*
EED	U90651, AF070418, AF078933, AF080227
EPHB2	L41939, AF025304
FZD8	n.a.*
GLI4	n.a.*
HNF4B	n.a.*
HOXA3	n.a.*
HOXA6	n.a.*
HOXB9	n.a.*
HOXC9	n.a.*

HOXD8	n.a.*
IHH	L38517
LMX1A	n.a.*
MEIS3	U68385
ORW2	n.a.*
PKHD1	n.a.*
RIEG2	n.a.*
SFRP2	AF017986
SOX10	n.a.*
TBX15	n.a.*
TBX18	AJ010278
TBX7	n.a.*
TFCOUP2	M62760, M64497, U60477
WNT14	AF028702
WNT15	AF028703
WNT3	n.a.*
WNT6	n.a.*
WNT7B	n.a.*
WNT8A	n.a.*
FGF2	NM_002006
LHX2	NM_004789
TBX6	NM_004608
ZIC3	NM_003413
ACVR2B	NM_001106
APC	NM_000038
BDNF	NM_001709
BMP1	NM_006129
BMPR1B	NM_001203
BMPR2	NM_001204
EDR2	NM_004427
FGF16	NM_003868
FGF3	NM_005247
FGF4	NM_002007
FGFR3	NM_000142 and NM_022965
FRZB	NM_001466
FZD7	NM_003507
GLI2	NM_005270
HNF3A	NM_004496
HOXB4	NM_024015
HOXB8	NM_024016
HOXC8	NM_022658
HOXD1	NM_024501
INHBB	NM_002193
LMX1B	NM_002316)
MADH3	NM_005902
PAX1	NM_006192
PAX9	NM_006194

PBX3	NM_006195
PROP1	NM_006261
PTCH2	NM_003738
RARG	NM_000966
RXRA	NM_002957
SFRP5	NM_003015
SOX1	NM_005986
TGFBR1	NM_004612
WNT2B	NM_004185
GSC	n.a.*

* "n.a." designates genes for which no meaningful sequences could be identified

Claims

1. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of genes associated with development according to one of the sequences taken from the group of Seq. ID No.1 to Seq. ID No.350 and sequences complementary thereto.

2. A nucleic acid comprising a sequence at least 18 base pairs in length of a segment of the chemically pretreated DNA of genes associated with diseases associated with development according to one of the sequences according to the genes ACCPN, ADFN, AFD1, AHO2, AIH3, AMCD1, AMCD2B, AMCN, AMCX1, AMDM, ANOP1, ASMD, ATD, AXIN1 AF009674, BDB1, BDC, BDE, BDMR, CHH, CHRD AF076612, CHX10, EED (U90651, AF070418, AF078933, AF080227), EPHB2 (L41939, AF025304), FZD8, GLI4,GSC, HNF4B, HOXA3, HOXA6, HOXB9, HOXC9, HOXD8, IHH (L38517), LMX1A, MEIS3 (U68385), ORW2, PKHD1, RIEG2, SFRP2 (AF017986), SOX10, TBX15, TBX18 (AJ010278), TBX7, TFCOUP2 (M62760, M64497, U60477), WNT14 (AF028702), WNT15 (AF028703), WNT3, WNT6, WNT7B, WNT8A, FGF2 (NM_002006), LHX2 (NM_004789), TBX6 (NM_004608), ZIC3 (NM_003413), ACVR2B (NM_001106), APC (NM_000038), BDNF (NM_001709), BMP1 (NM_006129), BMPR1B (NM_001203), BMPR2 (NM_001204), EDR2 (NM_004427), FGF16 (NM_003868), FGF3 (NM_005247), FGF4 (NM_002007), FGFR3 (NM_000142&NM_022965), FRZB (NM_001466), FZD7 (NM_003507), GLI2 (NM_005270), HNF3A (NM_004496), HOXB4 (NM_024015), HOXB8 (NM_024016), HOXC8 (NM_022658), HOXD1 (NM_024501), INHBB (NM_002193), LMX1B (NM_002316), MADH3 (NM_005902), PAX1 (NM_006192), PAX9 (NM_006194), PBX3 (NM_006195), PROP1 (NM_006261), PTCH2 (NM_003738), RARG (NM_000966), RXRA (NM_002957), SFRP5 (NM_003015), SOX1 (NM_005986), TGFBR1 (NM_004612), WNT2B (NM_004185) and sequences complementary thereto.

3. An oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at

least 9 nucleotides which hybridizes to or is identical to a chemically pretreated DNA of genes associated with diseases associated with development according to one of the Seq ID Nos 1 to 350 according to claim 1 or to a chemically pretreated DNA of genes according to claim 2 and sequences complementary thereto.

4. The oligomer as recited in Claim 3; wherein the base sequence includes at least one CpG dinucleotide.

5. The oligomer as recited in Claim 3, characterized in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.

6. A set of oligomers, comprising at least two oligomers according to any of claims 3 to 5.

7. A set of oligomers as recited in Claim 6, comprising oligomers for detecting the methylation state of all CpG dinucleotides within one of the sequences according to Seq. ID Nos. 1 through 350 according to claim 1 or a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto.

8. A set of at least two oligonucleotides as recited in Claim 3, which can be used as primer oligonucleotides for the amplification of DNA sequences of one of Seq. ID 1 through Seq. ID 350 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto and segments thereof.

9. A set of oligonucleotides as recited in Claim 8, characterized in that at least one oligonucleotide is bound to a solid phase.

10. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of claims 6 through 9 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) in a chemically pretreated genomic DNA according to claim 1 or a chemically pretreated DNA of genes according to claim 2.

11. A method for manufacturing an arrangement of different oligomers (array) fixed to a

carrier material for analyzing diseases associated with the methylation state of the CpG dinucleotides of one of the Seq. ID 1 through Seq. ID 350 and sequences complementary thereto and/or chemically pretreated DNA of genes according to claim 2, wherein at least one oligomer according to any of the claims 3 through 5 is coupled to a solid phase.

12. An arrangement of different oligomers (array) obtainable according to claim 11.

13. An array of different oligonucleotide- and/or PNA-oligomer sequences as recited in Claim 12, characterized in that these are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.

14. The array as recited in any of the Claims 12 or 13, characterized in that the solid phase surface is composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold.

15. A DNA- and/or PNA-array for analyzing diseases associated with the methylation state of genes, comprising at least one nucleic acid according to one of the preceding claims.

16. A method for ascertaining genetic and/or epigenetic parameters for the diagnosis and/or therapy of existing diseases or the predisposition to specific diseases by analyzing cytosine methylations, characterized in that the following steps are carried out:
- in a genomic DNA sample, cytosine bases which are unmethylated at the 5-position are converted, by chemical treatment, to uracil or another base which is dissimilar to cytosine in terms of hybridization behaviour;
- fragments of the chemically pretreated genomic DNA are amplified using sets of primer oligonucleotides according to Claim 8 or 9 and a polymerase, the amplificates carrying a detectable label;
- amplificates are hybridized to a set of oligonucleotides and/or PNA probes according to the Claims 6 and 7, or else to an array according to one of the Claims 12 through 15;
the hybridized amplificates are subsequently detected.

17. The method as recited in Claim 16, characterized in that the chemical treatment is car-

ried out by means of a solution of a bisulfite, hydrogen sulfite or disulfite.

18. The method as recited in one of the Claims 16 or 17, characterized in that more than ten different fragments having a length of 100 - 2000 base pairs are amplified.

19. The method as recited in one of the Claims 16 through 18, characterized in that the amplification of several DNA segments is carried out in one reaction vessel.

20. The method as recited in one of the Claims 16 through 19, characterized in that the polymerase is a heat-resistant DNA polymerase.

21. The method as recited in Claim 20, characterized in that the amplification is carried out by means of the polymerase chain reaction (PCR).

22. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are fluorescence labels.

23. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are radionuclides.

24. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are detachable molecule fragments having a typical mass which are detected in a mass spectrometer.

25. The method as recited in one of the Claims 16 through 21, characterized in that the amplificates or fragments of the amplificates are detected in the mass spectrometer.

26. The method as recited in one of the Claims 24 and/or 25, characterized in that the produced fragments have a single positive or negative net charge for better detectability in the mass spectrometer.

27. The method as recited in one of the Claims 24 through 26, characterized in that detection is carried out and visualized by means of matrix assisted laser desorption/ionization

mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

28. The method as recited in one of the Claims 16 through 27, characterized in that the genomic DNA is obtained from cells or cellular components which contain DNA, sources of DNA comprising, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, and all possible combinations thereof.

29. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to one of the Claims 3 through 5.

30. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of the Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of claims 6 through 9 for the diagnosis of diseases associated with development genes, in particular diseases related to homeobox containing genes (HOX), like diabetes and cancer, apoptosis related diseases, syndromes associated with congenital heart disease, epilepsy, diseases related to histone deacetylation, Currarino syndrome, diseases related with the development of the brain and limb girdle muscular dystrophy, dwarfism, and the like.

31. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of claims 6 through 9 for the therapy of diseases associated with development genes, in particular diseases related to homeobox containing genes (HOX), like diabetes and cancer, apoptosis related diseases, syndromes associated with congenital heart disease, epilepsy, diseases related to histone deacetylation, Currarino syndrome, diseases related with the development of the brain and limb girdle muscular dystrophy, dwarfism, and the like.

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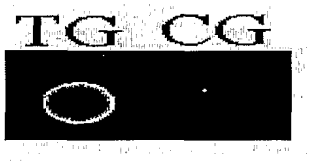
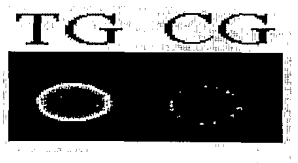
Fig 1



I



II

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2002 (03.01.2002)

PCT

(10) International Publication Number
WO 02/000927 A3

(51) International Patent Classification[7]: C12Q 1/68, B01J 19/00, G01N 33/483

(21) International Application Number: PCT/EP01/07536

(22) International Filing Date: 2 July 2001 (02.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 32 529.7 30 June 2000 (30.06.2000) DE
100 43 826.1 1 September 2000 (01.09.2000) DE

(71) Applicant *(for all designated States except US)*: **EPIGENOMICS AG** [DE/DE]; Kastanienallee 24, 10435 Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **OLEK, Alexander** [DE/DE]; Schröderstrasse 13/2, 10115 Berlin (DE). **PIEPENBROCK, Christian** [DE/DE]; Schwartzkopffstrasse 7 B, 10115 Berlin (DE). **BERLIN, Kurt** [DE/DE]; Marienkäferweg 4, 14532 Stahnsdorf (DE).

(74) Agents: **SCHOHE, Stefan** et al.; Boehmert & Boehmert, Pettenkoferstrasse 20-22, 80336 Munich (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) Date of publication of the international search report:
18 July 2002

(15) Information about Correction:
Previous Correction:
see PCT Gazette No. 12/2002 of 21 March 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH DEVELOPMENT BY MEANS OF ASSESSING THEIR METHYLATION STATUS

I

II

(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with diseases associated with development, to oligonucleotides and/or PNA/oligomers for detecting the cytosine methylation state of genes associated with diseases associated with development which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with diseases associated with development.

WO 02/000927 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT, _? 01/07536

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 B01J19/00 G01N33/483

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q B01J G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBL, MEDLINE, BIOSIS, WPI Data, PAJ, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BENDER CM ET AL.: "Roles of cell division and gene transcription in the methylation of CpG islands." MOLECULAR AND CELLULAR BIOLOGY, vol. 19, no. 10, October 1999 (1999-10), pages 6690-6698, XP002185033 page 6691, right-hand column, paragraph 3 -page 6692, left-hand column, paragraph 1 ---	1
X	TOKUHARA M ET AL.: "Molecular cloning of the human frizzled-6" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 243, 1998, pages 622-627, XP002185034 figure 1 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2001	08. 04. 02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ulbrecht, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL [Online] EBI; 15 November 1999 (1999-11-15) WATERSTON RH: "The sequence of Homo sapiens clone"" retrieved from HTTP://WWW.EBI.AC.UK/CGI-BIN/EMBLFETCH Database accession no. AC013731 XP002185035 the whole document	1,30,31
Y		3-29

X	DATABASE EMBL [Online] EBI; 2 May 2000 (2000-05-02) BOECKELMANN R ET AL.: "Gene expression in psoriatic skin." retrieved from HTTP://WWW.EBI.AC.UK/CGI-BIN/EMBLFETCH Database accession no. AF143346 XP002185036 the whole document	1,30,31
Y		3-29
T	& BÖCKELMANN R ET AL: "'Suprabasal overexpression of the hsBPB7 gene in psoriatic epidermis as identified by reverse transcriptase-polymerase chain reaction differential display model comparing Psoriasis plaque tissue with peritonsillar mucosa." AMERICAN JOURNAL OF PATHOLOGY, vol. 158, no. 2, February 2001 (2001-02), pages 367-372, page 369, left-hand column, paragraph 2 table 1	1

X	DATABASE EMBL [Online] EBI; 19 July 1999 (1999-07-19) KOHARA Y ET AL: "Caenorhabditis elegans cDNA clone:yk578b10" retrieved from HTTP://WWW.EBI.AC.UK/CGI-BIN/EMBLFETCH Database accession no. AV200626 XP002185759 the whole document	3

Y	WO 99 28498 A (OLEK ALEXANDER ;WALTER JOERN (DE); EPIGENOMICS GMBH (DE); OLEK SVE) 10 June 1999 (1999-06-10) cited in the application the whole document	1-31

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INTERNATIONAL SEARCH REPORT

International Application No
PCT, _? 01/07536

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 744 305 A (FODOR STEPHEN P A ET AL) 28 April 1998 (1998-04-28) cited in the application column 6, line 5 claims 1,8,15,20,26 figure 14	9,11-15
A	WO 99 29898 A (MAX PLANCK GESELLSCHAFT ;BERLIN KURT (DE); GUT IVO GLYNNE (DE); LE) 17 June 1999 (1999-06-17) page 10, line 19 -page 15, line 21	24-27
E	WO 01 77377 A (PIEPENBROCK CHRISTIAN ;BERLIN KURT (DE); EPIGENOMICS AG (DE); OLEK) 18 October 2001 (2001-10-18) claims 1,3-32 SEQ ID NO. 71	1,3-31
E	WO 01 68912 A (PIEPENBROCK CHRISTIAN ;BERLIN KURT (DE); EPIGENOMICS AG (DE); OLEK) 20 September 2001 (2001-09-20) claims 1,3-32 SEQ ID NO. 513	1,3-31
E	WO 01 68911 A (PIEPENBROCK CHRISTIAN ;BERLIN KURT (DE); EPIGENOMICS AG (DE); OLEK) 20 September 2001 (2001-09-20) claims 1,3-32 SEQ ID NO. 389	1,3-31
E	WO 01 77375 A (EPIGENOMICS AG) 18 October 2001 (2001-10-18) claims 1,3-32 SEQ ID NO. 205	1,3-31
E	WO 01 77376 A (EPIGENOMICS AG) 18 October 2001 (2001-10-18) claims 1,3-32 SEQ ID NO. 48	1,3-31
A	GRIGG G AND CLARK S: "Sequencing 5-methylcytosine residues in genomic DNA" BIOESSAYS, CAMBRIDGE, GB, vol. 16, no. 6, June 1994 (1994-06), pages 431-436, XP002106411 ISSN: 0265-9247 cited in the application the whole document	1-31

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/EP 01/07536

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 2, 30 and 31
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: 2 (part.9
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 3-31 (partly)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1, 3-31(part.)

Invention 2:

A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of a gene associated with development according to sequence SEQ ID NO. 3 or 4, oligonucleotides having a sequence of at least 9 nucleotides identical or hybridizing to said DNA; a set of said oligonucleotides; the use of said set for detecting SNPs or the methylation state of cytosines in said nucleic acid; an array or set of said oligonucleotides fixed to a carrier; a method of producing said array; a method for diagnosis and/or therapy of diseases or disease predispostion using said oligonucleotides by analysing cytosine methylations; a kit comprising said oligonucleotides and bisulfite; and the use of said nucleic acid, oligonucleotides, set of oligonucleotides, array or kit for the diagnosis or therapy of diseases associated with development genes.

2. Claims: 1, 3-31(part.)

Invention 2:

A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of a gene associated with development according to sequence SEQ ID NO. 3 or 4, oligonucleotides having a sequence of at least 9 nucleotides identical or hybridizing to said DNA; a set of said oligonucleotides; the use of said set for detecting SNPs or the methylation state of cytosines in said nucleic acid; an array or set of said oligonucleotides fixed to a carrier; a method of producing said array; a method for diagnosis and/or therapy of diseases or disease predispostion using said oligonucleotides by analysing cytosine methylations; a kit comprising said oligonucleotides and bisulfite; and the use of said nucleic acid, oligonucleotides, set of oligonucleotides, array or kit for the diagnosis or therapy of diseases associated with development genes.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 30 is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claim 31 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 2, 30 and 31

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

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Continuation of Box I.2

Claims Nos.: 2 (part.9

Claim 2 refers inter alia to DNA related to the genes designated by the acronyms: ACCPN, ADFN, AHO2, AMCD1, AMCD2B, AMCN, AMCX1, AMDM, ANOP1, ASMD, ATD, BDC, BDE, BDMR, CHH, GLI4, GSC, HNF4B, ORW2, and RIEG2. Said genes either can not be found or a number of apparently unrelated sequences are contained in the GenBank database are linked with said acronyms. Furthermore, the description of the present application does not provide any information beyond the acronyms with regard to said genes. Therefore, the subject-matter referring to said genes is unclear (Article 6 PCT) and cannot be searched.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2002 (03.01.2002)

PCT

(10) International Publication Number
WO 02/00927 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/EP01/07536

(22) International Filing Date: 2 July 2001 (02.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 32 529.7 30 June 2000 (30.06.2000) DE
100 43 826.1 1 September 2000 (01.09.2000) DE

(71) Applicant *(for all designated States except US)*: **EPIGENOMICS AG** [DE/DE]; Kastanienallee 24, 10435 Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **OLEK, Alexander** [DE/DE]; Schröderstrasse 13/2, 10115 Berlin (DE). **PIEPENBROCK, Christian** [DE/DE]; Schwartzkopffstrasse 7 B, 10115 Berlin (DE). **BERLIN, Kurt** [DE/DE]; Marienkäferweg 4, 14532 Stahnsdorf (DE).

(74) Agents: **SCHOHE, Stefan** et al.; Boehmert & Boehmert, Pettenkoferstrasse 20-22, 80336 Munich (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(48) Date of publication of this corrected version:
21 March 2002

(15) Information about Correction:
see PCT Gazette No. 12/2002 of 21 March 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH DEVELOPMENT GENES

I

II

(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with diseases associated with development, to oligonucleotides and/or PNA/oligomers for detecting the cytosine methylation state of genes associated with diseases associated with development which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with diseases associated with development.

WO 02/00927 A2